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PU.1 Is a Component of a Multiprotein Complex Which Binds an Essential Site in the Murine Immunoglobulin λ2-4 Enhancer

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B-cell-specific enhancers have been identified in the immunoglobulin λ locus 3′ of each constant-region cluster. These enhancers contain two distinct domains, λA and λB, which are essential for enhancer function. λB contains a near-consensus binding site for the Ets family of transcription factors. In this study, we have identified a B-cell-specific protein complex which binds the λB motif of the λ2-4 enhancer in vitro and appears necessary for the activity of the enhancer in vivo, since mutations in λB which prevent this interaction also eliminate enhancer function. This complex contains PU.1, a member of the Ets family, and a transcriptional activator whose expression is restricted to cells of the hematopoietic system with the exception of T lymphocytes. In addition, it contains a factor which binds specifically to a region adjacent to the PU.1 binding site. This factor cannot bind λB autonomously but appears to require interaction with the PU.1 protein to stabilize its association with the DNA. This complex may be identical or related to the PU.1/NF-EM5 complex which interacts with a homologous DNA element in the immunoglobulin κ 3′ enhancer.

The expression of murine immunoglobulin (Ig) heavy- and light-chain genes is tightly controlled in a cell-type- and developmental stage-specific fashion. This regulation occurs at two levels, transcription and recombination. Transcription of Ig genes is regulated by cell-type-specific promoter and enhancer elements (8, 59, 62). In addition, a lymphocyte-specific recombinase acts in a temporally regulated manner to recombine the germ line Ig loci, resulting in the creation of open reading frames which code for functional heavy- and light-chain protein molecules (13, 56). It has been suggested that the binding of regulatory factors to *cis*-acting transcriptional control regions may precede Ig gene rearrangement and may in fact effect the changes in the chromatin structure of these regions which allow access of the recombinase machinery (5, 14, 57, 70). In this manner, control of Ig gene rearrangement may be mediated by the tissue-specific and developmentally regulated expression of *trans*-acting promoter- and enhancer-binding factors in B cells. Therefore, enhancer and promoter elements may play an important role in the initiation of the recombination process as well as in the cell-type- and stage-specific control of the expression of functionally rearranged Ig genes.

The control elements which regulate Ig gene expression have been extensively studied. B-cell-specific enhancers have been identified and well characterized in the major J-C introns of both the heavy (2, 18, 38) and κ light-chain (46, 51) genes. These enhancers are modular in structure, consisting of multiple, often redundant binding sites for positive- and negative-acting nuclear factors. Some of these sites are shared among enhancers, while others are unique to a particular enhancer and may thus confer specificity of function. Complex protein-protein interactions and subtle variations in factor concentrations may affect enhancer activity differently in different cell types (9).

More recently, enhancers have been found 3′ of constant-region gene segments in both the heavy-chain (31, 45) and κ light-chain (32) loci. These enhancers are similar to the intronic enhancers in that they are modular in structure and bind to cell-type- and stage-restricted activating and silencing factors, but they appear to be regulated differently than their intronic counterparts (21, 33, 42, 48, 50). The specific roles played by each of these various enhancers in B-cell development and Ig gene expression have yet to be completely understood.

A search for enhancers in the J-C introns of the λ light-chain gene locus was unsuccessful (46), but subsequent studies identified two B-cell-specific enhancers, one 3′ of each λ constant-region cluster (23). These enhancers, termed $E_{\lambda 2\text{-}4}$ (15.5 kb 3′ of Cλ2Cλ4) and $E_{\lambda 3\text{-}1}$ (35 kb 3′ of Cλ3Cλ1) are 90% homologous over the minimal 229-bp region of $E_{\lambda 2\text{-}4}$ which has full cell-type-specific activity and are separated by over 100 kb in the mouse genome. They share no extensive homology with any of the previously described Ig enhancers.

Four functional domains were identified in $E_{\lambda 2\text{-}4}$ by deletion and mutation analysis of the enhancer in conjunction with a chloramphenicol acetyltransferase (CAT) reporter gene construct (55). Two of these domains, λA and λB, were absolutely essential for enhancer activity, while the others, λE1 and λE2, were required for optimal activity. Specific protein interactions with each of these domains were detected by DNase I footprinting and gel mobility shift analyses. Both λA and λB interacted with B-cell-specific factors.

Closer inspection of the λB site revealed a sequence homology to the purine-rich degenerate consensus binding site for the Ets proto-oncogene family of transcription factors (66) (Fig. 1A). The Ets family is composed of proteins which share homology to the 85-amino-acid DNA binding domain (the ETS domain) of Ets-1 (26), the cellular homolog of the v-*ets* oncogene product of the avian E26 retrovirus which causes erythroblastosis in chickens (30, 40). The Ets family includes human and murine Ets-1 (22, 68), human and murine Ets-2 (68), human Elk-1 and Elk-2 (52), human Erg (54), human Elf-1 (64), murine GABPα (28), murine PEA3 (69), murine PU.1/Spi-1/Sfpi-1/B1 (15, 19, 27, 35, 43), human Spi-B (53), murine and human Fli-1 (4), human SAP-1 (10), and *Drosophila* E74 (7). All of these proteins bind DNA in a

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sequence-specific manner, and a number of them have been shown to be transcriptional activators.

Using a nuclear extract from J558L, a λ1-producing myeloma, and electrophoretic mobility shift assays (EMSAs) and methylation interference assays, we have identified a multiprotein complex which contains PU.1 and interacts specifically with the λB site. This complex requires both the Ets binding site and an adjacent site for its formation. In transient transfection experiments employing a CAT reporter gene whose expression is driven by $E_{\lambda 2-4}$, mutations in λB which abolish the binding of this PU.1-containing complex also eliminate enhancer function.

MATERIALS AND METHODS

Preparation of nuclear extracts. Nuclear extracts were prepared by a protocol which is a modification of those of Schreiber et al. (58) and Dignam et al. (11). Routinely, 10^7 cells were harvested, washed twice with phosphate-buffered saline, and then transferred in a 1-ml volume to a 1.5-ml tube. Cells were pelleted for 30 s in a microcentrifuge and then resuspended in buffer A (10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.9], 10 mM KCl, 0.1 mM EDTA, 0.5 mM dithiothreitol [DTT], 1 μg of pepstatin A [Boehringer Mannheim] per ml, 2 μg of aprotinin [Boehringer Mannheim] per ml, 2 μg of leupeptin [Boehringer Mannheim] per ml, 0.1 mM phenylmethylsulfonyl fluoride [PMSF; Sigma]). After swelling for 15 min on ice, the cells were disrupted by homogenization in a 1-ml Dounce homogenizer with a type B pestle. Nuclei were pelleted in a microcentrifuge for 1 min at 4°C. The supernatant was removed, and the nuclear pellet was resuspended in buffer C (20 mM HEPES [pH 7.9], 0.4 M NaCl, 1 mM EDTA, 25% glycerol, 0.5 mM DTT, 1 μg of pepstatin A per ml, 2 μg of aprotinin per ml, 2 μg of leupeptin per ml, 0.1 mM PMSF) by dispersion with a micropestle. Nuclear protein was extracted by rotating the tube for 30 min at 4°C. After pelleting of the cellular debris (5 min in a microcentrifuge), the supernatant was dialyzed against 50 ml of buffer D (20 mM HEPES [pH 7.9], 100 mM KCl, 0.2 mM EDTA, 20% glycerol, 0.5 mM DTT, 1 μg of pepstatin A per ml, 2 μg of aprotinin per ml, 2 μg of leupeptin per ml, 0.1 mM PMSF) on a rotator at 4°C for 5 h. The dialysate was spun for 20 min in a microcentrifuge, aliquoted, and stored at −80°C. Protein content was determined by the Bradford assay (6) with a kit provided by Bio-Rad.

In vitro transcription and translation. A plasmid containing the full-length PU.1 cDNA (27) (a kind gift from R. Maki) was linearized at the *Xba*I site 3′ of the PU.1 coding sequence. Capped PU.1 mRNA was synthesized by in vitro transcription of 5 μg of this plasmid in the presence of 10 mM 7mG(5′)ppp(5′)G (New England Biolabs) and T3 RNA polymerase (Stratagene). The resulting RNA was treated with 10 U of RNase-free DNase I (Ambion) for 15 min at 37°C, then phenol-chloroform extracted, chloroform extracted, ethanol precipitated, and resuspended in 100 μl of H_2O. A 2-μl aliquot of this RNA was used to program a 50-μl rabbit reticulocyte lysate in vitro translation reaction. After 1 h at 30°C, the reaction mix was aliquoted and stored at −80°C.

EMSAs. EMSAs were performed as described by Singh et al. (61). Probes were synthesized as complementary oligonucleotide pairs containing *Xba*I and *Bam*HI ends (see Fig. 1B for sequence information). Annealed oligonucleotide pairs were cloned into *Spe*I-*Bam*HI-cut Bluescript II SK+ (Stratagene) to generate pEts, pmEts, pB, pBm1, pBm2, and pBm3 (Fig. 1B). The cloned oligonucleotides were sequenced for verification. Probes were generated by *Xba*I and *Hin*dIII digestion of 20 μg of the appropriate plasmid followed by a fill-in reaction of the 5′ overhangs with Klenow enzyme in the presence of [^{32}P]dCTP. The labeled probe was isolated by electrophoresis on 6% polyacrylamide, and the DNA was recovered by electrophoresis onto a DEAE membrane (NA45; Schleicher & Schuell). In addition to the sequences shown, all probes contained 36 bp of plasmid vector polylinker. Binding reaction mixtures contained 10 mM Tris (pH 7.5), 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 5% glycerol, 2 μg of poly(dI-dC) (Pharmacia), 0.45 μg of sonicated salmon sperm DNA, 0.45 μg of denatured sonicated salmon sperm DNA, 20,000 cpm of probe, and 8 μg of nuclear extract protein or 2 μl of in vitro-translated protein in a final volume of 20 μl. Annealed oligonucleotides (100 ng unless otherwise indicated) were used as competitor DNAs. Binding reaction mixtures were incubated for 20 min at room temperature and then electrophoresed on a 4% native polyacrylamide gel at 160 V for 2 h, using 0.5× TBES (1× TBES is 100 mM Tris-borate plus 2 mM EDTA [pH 8.6]) as the running buffer.

For antibody supershift analysis, 8 μg of nuclear extract protein was incubated with 2 μl of preimmune or immune serum in 1× binding buffer for 30 min on ice prior to the addition of the remaining components of the binding reaction mixture as described above.

Methylation interference. Methylation interference probes were derived from pλB, which was described previously (55). pλB was digested with *Hin*dIII and *Sst*I to generate the B upper-strand probe and with *Eco*RV and *Xba*I to generate the B lower-strand probe. The single 5′ overhang of each probe was filled in with Klenow fragment in the presence of [^{32}P]dCTP and [^{32}P]dATP. The labeled probes were isolated as described above. Methylation of the DNA probe, binding of the probe to protein, isolation of DNA-protein complexes, cleavage of the methylated DNA, and analysis of the DNA fragments were performed as described by Baldwin (1). Binding reaction mixtures contained 30 μg of nuclear extract protein or 5 μl of in vitro-translated protein and 5×10^5 cpm of methylated probe in a final volume of 20 μl. A control experiment indicated that at these conditions an excess of free probe was present. All other conditions were as described for the EMSA. Wet gels were exposed to film overnight at 4°C. Protein-DNA complexes were isolated in blocks of polyacrylamide, cast in 1% agarose, and electrophoresed onto a DEAE membrane. After cleavage with piperidine, the recovered fragments were electrophoresed on a 10% polyacrylamide–7 M urea sequencing gel.

CAT expression plasmid constructions. Plasmid pPR, described previously (55), contains the 229-bp *Pst*I-*Rsa*I fragment of $E_{\lambda 2-4}$, which retains full enhancer activity, cloned between the *Pst*I and *Sma*I sites of Bluescript SK−. Site-specific mutations were generated in $E_{\lambda 2-4}$ by an overlap extension polymerase chain reaction (PCR) (25) using pPR as a template, a pair of primers complementary to vector sequences flanking the enhancer, and a pair of complementary enhancer sequence primers containing the mutation of interest. The flanking primers which were used were M13R* (5′-CAGGAAACAGCTATGACCATG-3′) and oT7p20 (5′-TAATACGACTCACTATAGGG-3′). The specific primer pairs used to introduce mutations were E_λm1 (5′-GAGAAA TAAAAccttGTGAAACCAAG-3′ and 5′-CTTGGTTTCACa aggTTTTATTTCTC-3′), E_λm2 (5′-CATGGACTTGGTacgA CTTCC-3′ and 5′-GGAAGTcgtACCAAGTCCATG-3′), and E_λm3 (5′-CATGGACTTtcaTTCACTTCC-3′ and 5′-GGAA GTGAAtgaAAGTCCATG-3′). In the first set of PCRs,

oT7p20 was paired with the first mutation-specific primer listed in each group, while in a separate reaction, M13R* was paired with the second. The PCR products from these two reactions were isolated by electrophoresis in 1% agarose, and the DNA was recovered in a 20-μl volume by using Qiaex resin (Qiagen). One-microliter aliquots of the individual reaction products were combined, and this mix was used as the template for a second PCR which employed M13R* and oT7p20 as primers. The amplification product was digested with *Xba*I and *Hin*dIII, and the appropriately sized mutant enhancer fragment was recovered by 6% polyacrylamide gel electrophoresis followed by electrophoresis onto a DEAE membrane. The recovered DNA was cloned into the *Xba*I and *Hin*dIII sites of Bluescript II SK+ and sequenced to verify correctness. Enhancers with the desired mutations were then excised by digestion with *Spe*I and *Hin*dIII, recovered, and cloned into the *Spe*I and *Hin*dIII sites downstream of the CAT gene in OPC 1 (previously described as $OP_\lambda CAT1$ [23]) to make OPC 21 ($E_\lambda m1$), OPC 22 ($E_\lambda m2$), and OPC 23 ($E_\lambda m3$). OPC 20 was generated by cloning the *Spe*I-*Hin*dIII fragment of the unmutated pPR into the same site in OPC 1.

A CAT expression construct containing a multimer of the λB site was generated as follows. Complementary oligonucleotides comprising the 3′ portion of the λB site (Fig. 1A) were designed with *Bam*HI and *Bgl*II overhangs. They have the following sequences: BΔ5′ top, 5′-GATCCATAAAAG GAAGTGAAACCAAGA-3′; and BΔ5′ bottom, 5′-GATCTC TTGGTTTCACTTCCTTTTATG-3′. The oligonucleotides were kinase treated, annealed, and allowed to ligate to each other for 30 min at 15°C, after which time the ligase was inactivated by heating at 65°C for 20 min. The multimerized oligonucleotides were then digested with *Bam*HI and *Bgl*II to ensure that all concatemers contained only head-to-tail junctions. After digestion, the multimers were separated on a 6% polyacrylamide gel, and tetramers of BΔ5′ were isolated. The tetramer DNA was ligated into *Bam*HI-cut TKCAT (gift of M. Atchison), which contains cloning sites upstream of the herpes simplex virus thymidine kinase (TK) promoter driving CAT expression. A construct containing a single copy of the BΔ5′ DNA cloned into the *Bam*HI site of TKCAT was similarly produced. Structures of these constructs were verified by sequencing.

Transient transfection and CAT assays. The J558L murine myeloma cell line (obtained from S. Morrison [41]) was cultured in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum (HyClone), 1 mM sodium pyruvate, 50 μM β-mercaptoethanol, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Cells were grown to a density of 5×10^5 to 10×10^5 cells per ml and transfected by the DEAE-dextran method as follows. A total of 10^7 cells were washed twice with TS buffer (137 mM NaCl, 5 mM KCl, 0.4 mM Na_2HPO_4, 25 mM Tris, 1 mM $MgCl_2$, 0.7 mM $CaCl_2$ [pH 7.4]) and then resuspended in 1.5 ml of TS containing 5 μg of CAT reporter construct DNA, 1 μg of β-galactosidase reporter plasmid (pMC1924 [39]), and 0.25 mg of DEAE-dextran (Pharmacia) per ml. After incubation for 20 min at room temperature, 15 ml of Dulbecco's modified Eagle's medium containing 0.1 M chloroquine diphosphate was added, and the cells were transferred to a CO_2 incubator at 37°C for 1 h. The transfected cells were then pelleted and resuspended in 40 ml of tissue culture medium. After 44 to 48 h, cell lysates were prepared from washed cells essentially as described by Gorman et al. (20). An aliquot of lysate was removed for determination of β-galactosidase activity (39), and the remainder was heated to 60°C for 10 min, chilled on ice for 5 min, and then centrifuged at 15,000 × *g* for 10 min. CAT assays were performed as described previously (20). The volume of lysate used in each assay was normalized to β-galactosidase activity to control for variation in transfection efficiencies. Regions of the thin-layer chromatography plate containing unacetylated and acetylated [^{14}C]chloramphenicol were excised, and radioactivity was quantitated by liquid scintillation counting. All CAT assay data reported represent the average of at least three independent transfections.

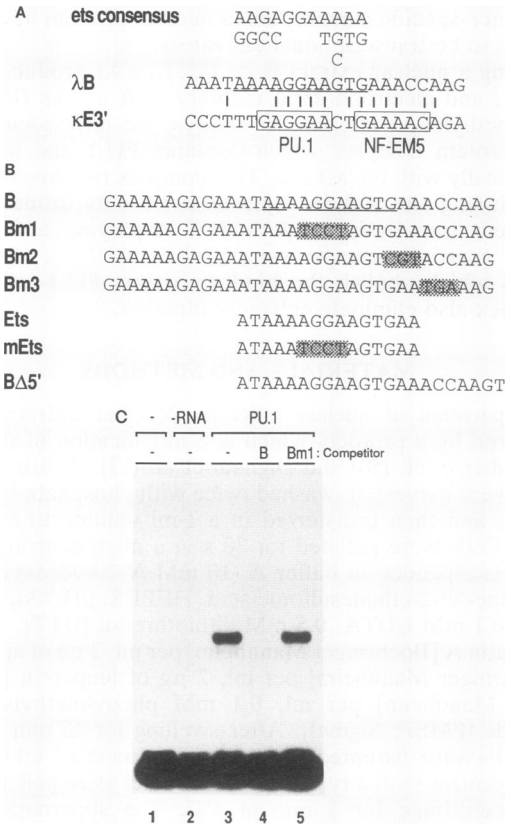

FIG. 1. (A) Nucleotide sequence of the λB site from $E_{\lambda 2-4}$ and alignment with the degenerate Ets consensus binding sequence and the homologous region of κE3′ (42). Nucleotides in λB which conform to the Ets consensus sequence are underlined. The identified binding sites for PU.1 and NF-EM5 on κE3′ are boxed. (B) DNA sequence of probes used in EMSAs. B is the full λB DNase I footprint. Bm1, Bm2, and Bm3 are identical to B except for the indicated mutations. Ets is a smaller probe containing just the Ets consensus region of the λB site, and mEts is a mutant form of this probe. BΔ5′ is the oligonucleotide which was multimerized for functional studies of the λB site (see Fig. 8). (C) EMSA using B as a probe. Lanes: 1, no protein; 2, 2 μl of control in vitro translation reaction mixture, which contained no RNA; 3 to 5, 2 μl of in vitro-translated PU.1. Lanes 4 and 5 contain 100 ng of the indicated double-stranded oligonucleotides as unlabeled competitors.

RESULTS

PU.1 binds specifically to the λB domain. Since the λB domain contains an Ets consensus binding site (Fig. 1A), and since this site is similar to a site in the κ E3′ enhancer (κE3′) which has been shown to bind PU.1 (50) (Fig. 1A), a tissue-specific Ets family member expressed in B cells, we

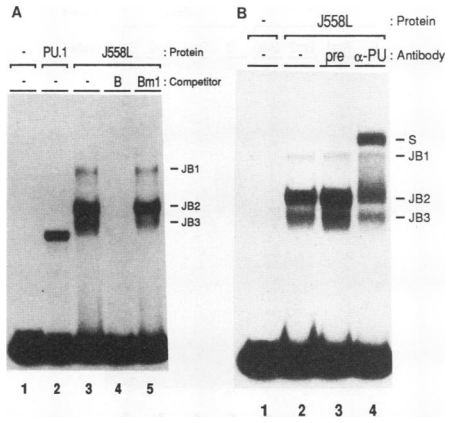

FIG. 2. EMSA using J558L nuclear extract and the B probe. (A) Analysis of complexes formed on the B probe from proteins contained in the J558L nuclear extract. Lanes: 1, no protein; 2, 2 μl of in vitro-translated PU.1; 3 to 5, 8 μg of J558L nuclear extract. Lanes 4 and 5 contain 100 ng of the indicated double-stranded oligonucleotides as competitors. JB1, JB2, and JB3 indicate the specific complexes formed by J558L nuclear extract on the B probe. (B) Anti-PU.1 antibody supershift assay with the B probe. Lanes: 1, no protein. 2 to 4, 8 μg of J558L nuclear extract; 3, 2 μl of preimmune rabbit serum; 4, 2 μl of rabbit anti-PU.1 antiserum. S indicates a supershifted complex which appears upon addition of anti-PU.1 antiserum.

decided to test PU.1's ability to bind this site in an EMSA. The probe (B; Fig. 1B) used in this assay corresponds to the entire λB site as determined by DNase I footprinting (55). PU.1 protein generated by in vitro translation was able to bind the B probe (Fig. 1C, lane 3). The appearance of a shifted complex was dependent on the presence of PU.1 mRNA in the in vitro translation reaction, as the control reaction with no RNA did not give rise to this complex (lane 2). In addition, the complex that was formed between PU.1 and the λB site was specific for the Ets binding site core, as the complex was effectively competed for by the addition of cold, double-stranded B (lane 4) but not Bm1 (lane 5), in which the central AGGA was altered to TCCT (Fig. 1B). Thus, PU.1 specifically recognizes the λB site of $E_{\lambda 2-4}$; the binding is dependent on the conserved central core of the Ets consensus sequence.

The three specific complexes which are formed by J558L nuclear extract with the λB probe require the Ets core. We have previously shown that three specific complexes are formed from J558L (derived from a λ1-producing myeloma) nuclear extract with the λB probe (55). To determine the importance of the Ets consensus binding site for the formation of these complexes, we used an EMSA with the B probe and oligonucleotides B and Bm1 as competitors. When J558L nuclear extract was incubated with radiolabeled B probe, the expected three complexes, referred to as JB1, JB2, and JB3, were formed (Fig. 2A, lane 3). All three of these complexes require the Ets core for binding, as they are efficiently competed for by cold (unlabeled) B (lane 4) but not by cold Bm1 (lane 5) DNA.

We expected PU.1 to be present in the J558L nuclear extract (although this had not been formally tested) and expected it to bind to the B probe (Fig. 1C). Therefore, it was somewhat surprising that none of the J558L complexes comigrated with that generated by recombinant PU.1 protein (Fig. 2A; compare lanes 2 and 3). All three of the complexes had lower mobilities in the native gel than did the PU.1 complex. There are a number of possibilities which could account for this observation, including the presence of a modified form of PU.1 in the J558L nuclear extract, the presence of PU.1 in a multiprotein complex, or the absence of DNA-binding-competent PU.1 in the J558L extract.

Complex JB2 contains PU.1. To determine whether the PU.1 protein was in fact present in any of the J558L complexes, we used a rabbit antiserum raised against a peptide in the amino terminus of PU.1 (50) and a gel supershift assay. Binding of the antibody to a PU.1-containing complex should cause an additional shift of that complex to a lower mobility in the native gel. While preincubation of J558L nuclear extract with preimmune rabbit serum had little effect on the complexes formed with B, preincubation with the anti-PU.1 antiserum diminished the intensity of the JB2 band and led to the appearance of a new band, S (Fig. 2B, lanes 3 and 4), thereby demonstrating the presence of PU.1 (or an antigenically related protein) in this complex. Complexes JB1 and JB3 were not significantly affected by the addition of anti-PU.1 antiserum.

Our results indicate that PU.1 is present in J558L nuclei in a form which is capable of interaction with the antiserum but appears in a DNA-protein complex whose mobility differs from that of the complex formed by the recombinant protein. It is not clear from this experiment whether the PU.1 which exists in J558L cells binds to λB in a complex with an additional protein(s) or whether it is a modified form which gives rise to a complex of altered mobility. These possibilities are addressed in the experiments described below.

Sequences in addition to the Ets site are required for the formation of JB2. We favored the idea that JB2 contained not only PU.1 but at least one additional protein, resulting in a complex of lower mobility than PU.1 alone. To determine whether we could detect both free PU.1 in the J558L nuclear extract, and additional sequence requirements for the formation of JB2, we used a shorter segment of λB which encompassed only the Ets motif as a probe in an EMSA. This probe, Ets (Fig. 1B), should be capable of binding Ets family proteins, including PU.1, but multiprotein complexes which require flanking sequences will not be formed. Multiprotein complexes which result from the binding of a single factor to the Ets site, with additional proteins added solely through protein-protein interactions, will be detected by the probe. Recombinant PU.1 bound specifically to the Ets probe (Fig. 3, lanes 2 to 4). While the Ets probe could compete for binding of PU.1 (lane 3), a probe with a four-base mutation in the Ets core, mEts (Fig. 1B), could not (lane 4). Two complexes were formed on the Ets probe with proteins from J558L nuclear extract (lane 5). Both of these complexes were sequence specific, requiring the AGGA tetranucleotide core of the Ets consensus sequence, as determined by competition with wild-type and mutant oligonucleotides (lanes 6 and 7). Importantly, one of these complexes exhibited an electrophoretic mobility identical to that of the in vitro-translated PU.1 (compare lanes 2 and 5). This complex supershifted upon addition of anti-PU.1 antiserum (12), thereby confirming that the native PU.1 in J558L nuclei has the same mobility in EMSAs as the cloned protein does.

Comparison of the relative mobilities of complexes formed on the B and Ets probes suggests that the slowest-migrating complex which forms on the Ets probe is identical to complex JB1 which is formed on the B probe. It is apparent that this complex requires only the Ets motif for its formation. It is not clear whether it is a complex of more than one protein factor. Complex JB2, which contains PU.1, is not

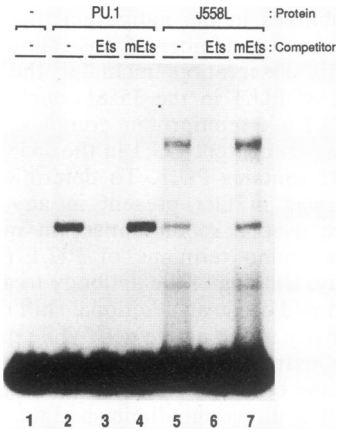

FIG. 3. EMSA using the Ets probe. Analysis of the complexes formed on the minimal Ets consensus region of the λB site from proteins contained in J558L nuclear extract is shown. Lanes: 1, no protein; 2 to 4, 2 μl of in vitro-translated PU.1; 5 to 7, 8 μg of J558L nuclear extract. Lanes 3, 4, 6, and 7 contain 100 ng of the indicated double-stranded oligonucleotides as unlabeled competitors for protein binding.

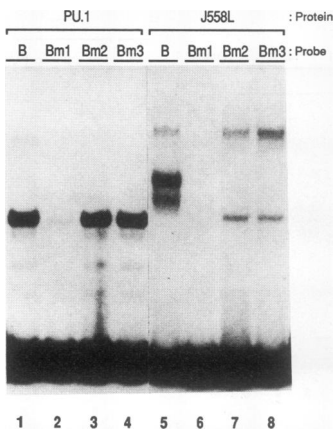

FIG. 4. EMSA using a panel of mutant B probes. Analysis of the binding site requirements of complexes formed on B from proteins contained in J558L nuclear extract is shown. Lanes: 1 to 4, 2 μl of in vitro-translated PU.1; 5 to 8, 8 μg of J558L nuclear extract. Identical amounts of similar probe preparations were used. Probes B, Bm1, Bm2, and Bm3 are depicted in Fig. 1B. B is the wild-type λB sequence. Bm1 contains a four-base mutation in the highly conserved core of the Ets consensus of λB. Bm2 and Bm3 contain adjacent 3-bp mutations in the region of λB which is 3′ of the PU box and has homology to a 6-bp binding site for NF-EM5 in κE3′ (Fig. 1A).

ormed on the Ets probe, even though PU.1 is able to bind this sequence, suggesting that additional factors, which bind λB sequences outside of the Ets consensus sequence, might participate in the formation of this complex. It is important to note that complex JB3 does not form on this probe either. Thus, it also requires sequences in addition to the Ets site for its formation.

Complex JB2 contains a factor which requires a sequence 3′ of the PU box for binding. Our results suggest that JB2 is a multicomponent complex composed of the DNA probe, PU.1, and at least one additional protein. Such a multiprotein complex has recently been described for a related element in κE3′, where PU.1 binds to a bipartite site in conjunction with a second factor, NF-EM5, which binds adjacent to the PU box (50). Since the DNA sequence 3′ of the PU box in λB was highly homologous to the NF-EM5 binding site in κE3′ (Fig. 1A), we decided to test whether this sequence was also required for the formation of JB2 on the B probe. Two DNA probes which consisted of the λB sequence with each half of the putative NF-EM5 binding site mutated (Bm2 and Bm3; Fig. 1B) were made. These particular mutations were shown to abolish NF-EM5 binding when introduced into κE3′ (50).

An EMSA was carried out by using this series of probes, which are identical in length, to allow a direct comparison of the complexes formed. Probes B and Bm1, which contains a four-base mutation in the Ets binding site core (Fig. 1B), were included along with Bm2 and Bm3. Recombinant PU.1 bound equally well to B, Bm2, and Bm3 (Fig. 4, lanes 1, 3, and 4), indicating that the two mutations 3′ of the PU box had no effect on PU.1 binding. In contrast, PU.1 bound only very weakly to Bm1 (lane 2). When J558L nuclear extract was incubated with Bm2 and Bm3, two complexes were formed (lanes 7 and 8). The faster-migrating of the two complexes had a mobility identical to that of the recombinant PU.1 (compare lanes 3 and 4 with lanes 7 and 8). This complex is indeed PU.1, since it is supershifted upon addition of the anti-PU.1 antiserum (12). The slower-migrating complex had a mobility identical to that of JB1. The pattern of complex formation on Bm2 and Bm3 is identical to the pattern of complex formation on the smaller Ets probe (Fig. 3). Importantly, complexes JB2 and JB3 cannot form with probes which contain mutations in, or lack, the putative binding site 3′ of the PU box, indicating that this site, too, is required for the formation of these complexes. These results support the hypothesis that complex JB2 consists of multiple proteins, with one protein, PU.1, binding to the Ets motif and a second protein(s) binding to the site 3′ of the Ets motif. This observation is reminiscent of the heterodimeric complex of PU.1 and NF-EM5, which forms with the highly homologous site in κE3′.

It is important to note that the protein which binds to the 3′ portion of the λB site in JB2 cannot do so stably in the absence of PU.1 binding (Fig. 4, lane 6). In addition, since no complex representing free PU.1 is formed with the B probe, it is clear that this 3′ binding factor is in excess, and the formation of the multimeric complex is favored.

JB2 proteins bind λB cooperatively. It is apparent from Fig. 4 that the amount of PU.1-containing complex formed from J558L nuclear extract is much greater on the B probe, where both the Ets and NF-EM5 sites are present (Fig. 4, lane 5; JB2) than on Bm2 or Bm3, where only the Ets site is present (Fig. 4, lanes 7 and 8; PU.1). This finding suggests that the factors which comprise JB2 might bind to λB in a cooperative fashion. We tested this hypothesis by EMSA in which we used equimolar amounts of B, Bm1, and Bm2 to compete for the binding of PU.1 and JB2 proteins to the B probe (Fig. 5). As expected, both B and Bm2 competed effectively for the binding of PU.1 (Fig. 5B, lanes 14 and 20), while Bm1 did not (lane 17). This finding supports our previous conclusion that PU.1 binds equally well to B and Bm2 but does not bind well to Bm1 (Fig. 4, lanes 1 to 3). On the other hand, while B competed effectively for the formation of JB2 (Fig. 5A, lane 4), an identical amount of Bm2 failed to fully compete for the formation of this complex (lane 10). In fact, more than 10 times as much Bm2 was required to achieve a level of competition similar to that of B (compare lanes 3 and 10).

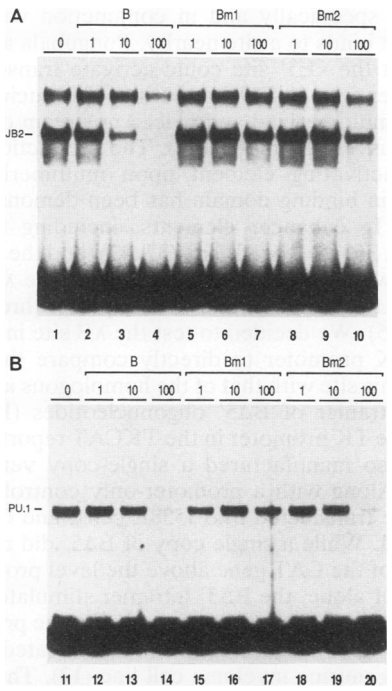

FIG. 5. Competition assay for cooperative binding. (A) EMSA using 8 μg of J558L nuclear extract and 20,000 cpm of B probe. Competitors are B (lanes 2 to 4), Bm1 (lanes 5 to 7), and Bm2 (lanes 8 to 10) and were added in the amounts indicated. 1, 10, and 100 indicate 1-, 10-, and 100-fold molar excesses of competitor over probe DNA. (B) EMSA using 2 μl of in vitro-translated PU.1, 20,000 cpm of B probe, and competitors in the amounts listed above.

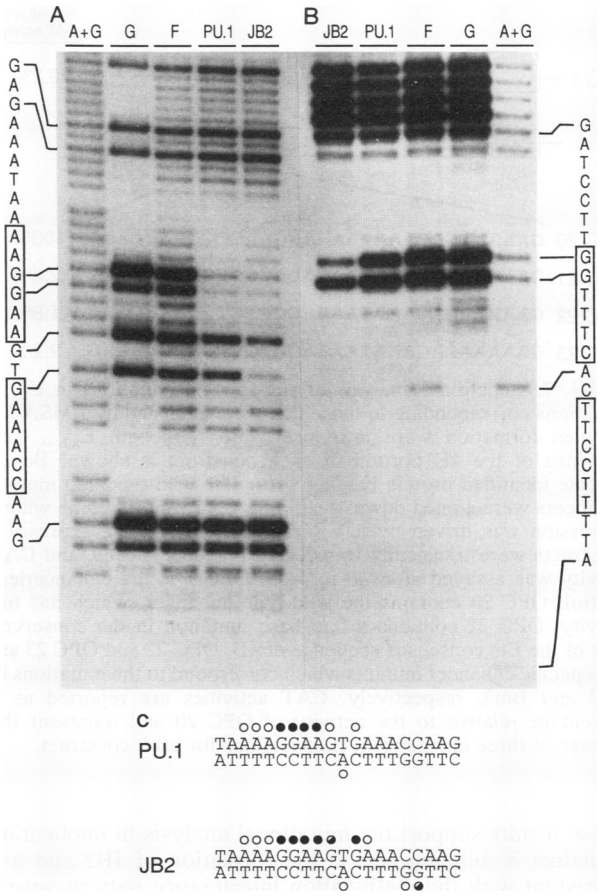

FIG. 6. Methylation interference assay of the λB site. (A and B) Analysis of the upper (A) and lower (B) strands of λB. Complexes were isolated and analyzed individually. PU.1 corresponds to the complex formed by in vitro-translated PU.1 on the λB probe. JB2 corresponds to the PU.1-containing complex formed from J558L nuclear extract on λB. F indicates unretarded probe recovered from the free-probe region of the native gel. A+G and G are the corresponding sequencing ladders. The probe sequence is shown for orientation; the PU.1 and 3′ binding sites are boxed. (C) Summary of the DNA contact sites for PU.1 and JB2. Closed circles indicate G and A residues whose methylation results in a complete loss of complex formation, partially filled circles indicate G residues whose methylation results in a substantial decrease in complex formation, and open circles indicate G and A residues whose methylation results in a modest decrease in complex formation.

Thus, PU.1 binds cooperatively to the bipartite λB site when the other components of JB2 are present.

Methylation interference analysis of J558L complexes formed with the B probe. We performed a methylation interference assay on the λB DNA to determine the sites of protein-DNA contact upon complex formation. Methylated λB DNA was isolated from JB2 and compared with similarly methylated λB DNA which ran as free probe in the native gel. PU.1 generated by in vitro translation was assayed in the same manner. The results of the methylation interference assay are shown in Fig. 6. Both strands of the λB probe were analyzed, although there was a paucity of informative residues on the lower strand (Fig. 6B). Treatment of the DNA probe with dimethyl sulfate results in methylation of G residues at the N-7 position in the major groove and A residues at the N-3 position in the minor groove. Bases which are in close contact with protein upon complex formation, and whose methylation interferes with protein binding, are revealed as bands present in the free-probe lane but diminished or absent in the complexed-probe lane. The autoradiographic data are summarized in Fig. 6C.

Analysis of the upper strand of λB (Fig. 6A) demonstrates that in vitro-translated PU.1 makes close contacts with the conserved GGAA tetranucleotide of the Ets consensus sequence. Methylation of any one of these bases drastically impairs the ability of PU.1 to bind this site (Fig. 6C; filled circles indicate strong interference). In addition, methylation of any one of the three A's upstream or two G's downstream of this tetranucleotide modestly inhibits PU.1 binding (Fig. 6C; open circles indicate weak interference). A similar pattern of contacts is revealed by the analysis of JB2, but the effect of methylation at either of the two G's 3′ of the PU box is much more drastic. Methylation of the first of these, which lies just outside of the PU box, clearly has a greater effect on JB2 formation than on PU.1 binding. Methylation of the second G, which is the first nucleotide of the putative NF-EM5 binding site (50), completely abolishes formation of JB2 but only partially inhibits formation of the PU.1 complex.

The lower strand of λB is relatively free of G's and A's but does allow an examination of the two G's at the distal end of the 3′ binding site (Fig. 6B). This analysis demonstrates that these two bases are not contacted in the complex formed with in vitro-translated PU.1, as predicted by the mutational analysis. The opposite is true for JB2; although the effect is more pronounced at the most distal of the pair, methylation of either of these G's clearly inhibits the formation of JB2.

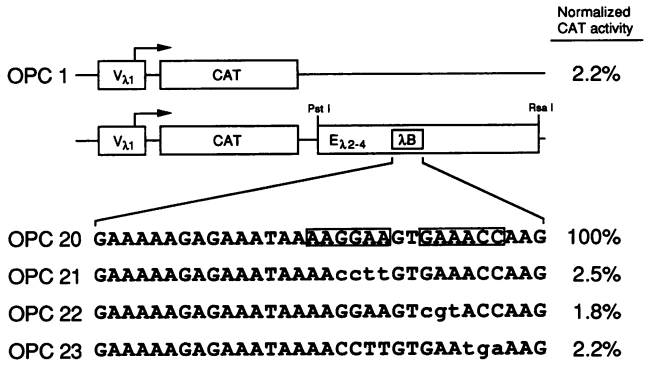

FIG. 7. Functional analysis of site-specific mutations in $E_{\lambda 2\text{-}4}$. Mutations corresponding to those that were analyzed by EMSA for complex formation were introduced into full-length $E_{\lambda 2\text{-}4}$. The sequence of the λB portion of each construct is shown. Boxes indicate identified protein binding sites. The wild-type and mutant enhancers were cloned downstream of a CAT reporter gene whose expression was driven by a $V_{\lambda 1}$ promoter. The CAT expression constructs were transiently transfected into J558L cells, and CAT activity was assayed after 40 to 44 h. OPC 1 is the enhancerless control. OPC 20 contains the wild-type enhancer, which has full activity. OPC 21 contains a four-base mutation in the conserved core of the Ets consensus sequence of λB. OPC 22 and OPC 23 are site-specific enhancer mutants which correspond to the mutations in Bm2 and Bm3, respectively. CAT activities are reported as a percentage relative to the activity of OPC 20 and represent the average of three independent transfections for each construct.

These results support the mutational analysis in implicating a distinct 3′ binding site in the formation of JB2 and are consistent with the methylation interference pattern generated by PU.1 and NF-EM5 on κE3′ (50).

Functional analysis of the PU.1 and 3′ binding sites. In an effort to establish a correlation between the in vitro formation of complexes on the λB site and the in vivo activity of $E_{\lambda 2\text{-}4}$, we used a CAT reporter construct transfected into and transiently expressed in J558L cells. The reporter construct consisted of the bacterial CAT gene, whose expression was driven by the $V_{\lambda 1}$ promoter, with the fully active 229-bp *Pst*I-*Rsa*I fragment of $E_{\lambda 2\text{-}4}$ downstream of the CAT coding sequences. Site-specific mutations, which correspond to the mutations introduced into the B probe for the gel shift assays described above, were inserted into the enhancer in the context of the full reporter construct. Constructs were transfected into J558L cells and analyzed for CAT activity, which is reported as a percentage of the activity of the wild-type enhancer construct (Fig. 7). Mutation of the Ets core, which abolishes the formation of all three complexes in vitro, reduces CAT activity to the level of the enhancerless construct (Fig. 7, OPC 21; compare with Fig. 4, lane 6). Mutation of either half of the 3′ binding site, which is required for the formation of complexes JB2 and JB3, also reduces CAT activity to the level of the enhancerless construct (Fig. 7, OPC 22 and OPC 23; compare with Fig. 4, lanes 7 and 8).

Both the Ets site and the site 3′ of it are required for enhancer function in vivo. Therefore, the in vivo activity of $E_{\lambda 2\text{-}4}$ correlates with the binding of the PU.1-containing complex, JB2, to the λB site DNA in vitro; the base pairs which are required for the formation of this complex in vitro are the same as those required for enhancer activity in vivo.

A multimerized λB site has activating potential. The λB site appears quite similar to the homologous site from κE3′: it binds PU.1 specifically and in conjunction with a second factor which binds to a site nearby. Pongubala and Atchison showed that the κE3′ site could activate transcription of a CAT reporter gene in S194 (a murine κ-producing myeloma) cells when multimerized and placed upstream of the herpes simplex virus TK promoter (48). The production of a powerful transactivating element upon multimerization of a single protein binding domain has been demonstrated for a number of Ig enhancer elements, including the octamer element (17, 36, 63), the κB site (37, 47), and the μE3 site (3). Previously we had shown that a trimer of the λB site could not activate transcription of a CAT reporter through the $V_{\lambda 1}$ promoter (55). We decided to test the λB site in conjunction with the TK promoter to directly compare the functional activity of this site with that of the homologous κE3′ site. We cloned a tetramer of BΔ5′ oligonucleotides (Fig. 1B) upstream of the TK promoter in the TKCAT reporter construct (48). We also manufactured a single-copy version of this construct. Along with a promoter-only control, these constructs were transfected into J558L cells, and CAT activity was assayed. While a single copy of BΔ5′ did not stimulate expression of the CAT gene above the level provided by the TK promoter alone, the BΔ5′ tetramer stimulated transcription of the reporter 14-fold compared with the promoter-only construct (Fig. 8). This result was duplicated in S194, a κ-expressing murine myeloma cell line (12). Thus, the truncated λB site, when multimerized, can activate transcription from the TK promoter, just as the homologous region from κE3′ can.

DISCUSSION

In this report, we have demonstrated that a B-cell-specific protein complex binds to the λB motif of $E_{\lambda 2\text{-}4}$ in vitro and appears necessary for the in vivo activity of this enhancer, since mutations in λB which prevent this interaction also eliminate enhancer function. This complex contains PU.1, a member of the Ets proto-oncogene family which is a transcriptional activator whose expression is restricted to cells of the hematopoietic system with the exception of T lymphocytes (15, 27, 53). Moreover, this complex appears to contain an additional factor which binds specifically to a region adjacent to the PU.1 binding site. This additional factor cannot bind the λB motif autonomously; it appears to require direct interaction with the PU.1 protein to stabilize its association with the DNA. The binding of PU.1 to the λB site is facilitated by the presence of this additional factor when its 3′ DNA binding site is present. The ternary complex which is formed may be identical or related to the PU.1/NF-EM5 complex which interacts with a homologous DNA element in the Ig κ 3′ enhancer (50).

The Ets gene family consists of a number of recently identified transcription factors, all of which bind specifically to closely related DNA sequences. We have not ruled out the possibility that other members of this family, either B-cell specific or otherwise, might bind the λB sequence in vivo and serve a function in regulating the activity of the λ enhancers. Indeed, at the present time, we have not been able to distinguish between the in vitro binding activities of JB2 and JB3. All of the mutations which abolish the formation of one complex have the same effect on the other (Fig. 4). Therefore, the relative importance of each of these complexes for in vivo enhancer activity has yet to be determined. We are currently attempting to conclusively identify all of the factors which interact to form complexes

JB2 and JB3, and we hope that their characterization will allow us to address this question.

In an EMSA, JB2 is clearly supershifted by the addition of anti-PU.1 antiserum, identifying PU.1 as a component of this complex (Fig. 2B, lane 4). The conserved core of the Ets motif is required for PU.1 binding, as determined by mutation of this site in binding studies (Fig. 4, lanes 2 and 6). Furthermore, although mutation of a 6-bp sequence 3′ of the Ets site had no effect on PU.1 binding, it prevented the formation of JB2, suggesting the presence of a second DNA-binding factor in this complex (Fig. 4, lanes 3, 4, 7, and 8). Methylation interference analysis supports the model of a bipartite binding element in λB (Fig. 6C); methylation of individual nucleotides in either the PU box or the 3′ binding site interferes with the formation of JB2. The extent of contacts revealed for JB2 by methylation interference is similar to that reported for the PU.1/NF-EM5 complex bound to κE3′ (50). Preliminary experiments indicate that JB2 has an electrophoretic mobility identical to that of the PU.1/NF-EM5 complex when the two complexes are formed with probes of equivalent size (12). It is very likely that these two B-cell-specific complexes are the same and that JB2 contains NF-EM5 as well as PU.1. NF-EM5 is a B-cell-specific protein whose interaction with κE3′ in conjunction with PU.1 is required for the activity of that enhancer (50). Likewise, the binding of JB2 to the λB site is correlated with the activity of $E_{\lambda 2-4}$.

$E_{\lambda 2-4}$ and $E_{\lambda 3-1}$ are highly homologous and are thought to be regulated by similar, if not identical, mechanisms (55). These enhancers exhibit a substantial degree of sequence similarity in the region of the λB footprint, with only two nucleotide differences over the 32-bp stretch. Both of these variations occur within the Ets motif; the two enhancers are identical in sequence at the 3′ binding site. One of the differences occurs at the first nucleotide of the PU box, where neither enhancer conforms to the Ets consensus sequence. The other occurs at the first base pair 3′ of the PU box. Here, the G of $E_{\lambda 2-4}$ is altered to a C in $E_{\lambda 3-1}$. This change is consistent with the Ets consensus sequence. In fact, a C is found at this position in the κE3′ site. We have not analyzed the λB sequence from $E_{\lambda 3-1}$ for interaction with PU.1 or for complex formation with J558L nuclear extract, but we expect that its binding properties will be equivalent to those of $E_{\lambda 2-4}$, given the similar natures of the enhancers.

Previously described PU.1 binding sites consisted of the hexanucleotide sequence, 5′-GAGGAA-3′, termed the PU box (27, 44, 48). The λB element contains the sequence 5′-AAGGAA-3′, which binds PU.1 avidly. The single nucleotide difference, which in λB does not conform to the described Ets consensus sequence, occurs at a position previously shown to be in close contact with the PU.1 protein (27, 50). Our methylation interference data suggest that PU.1 retains a close contact with this position in λB. In addition, PU.1 is able to bind, albeit extremely weakly, to the Bm1 probe, in which the core of the PU box has been mutated (Fig. 4, lane 2) (12). These results indicate that nucleotides in addition to those that comprise the PU box may be important determinants for the specificity of PU.1 binding. Others have recently reported that DNA sequences which flank the PU box may play a role in the specific interaction of PU.1 with its recognition element (67). Furthermore, it has been shown that nucleotides at the periphery of the Ets consensus sequence are critical for distinguishing between high-affinity binding sites for some members of the Ets family (66).

The binding of a factor contained in JB2 to the 3′ binding site is strictly dependent on the presence of λB-bound PU.1, indicating that direct protein-protein interaction is probably important for the formation of this complex. Such an interaction has been described for κE3′, where NF-EM5 binds its recognition element only when PU.1 is present and capable of binding to the PU box (50). A domain of the PU.1 protein which is required for the interaction between these two proteins has been identified; the phosphorylation of a single serine (Ser-148) within this domain is essential for this interaction (49). When this domain is deleted or when phosphorylation of Ser-148 is prevented, PU.1 is no longer able to recruit NF-EM5 to its downstream binding site (49, 50). Important protein-protein interactions and cooperative binding to composite DNA elements have been described for other members of the Ets family, including Elk-1 and SAP-1, which interact with serum response factor to bind to serum response elements (10, 24), Ets-1, which interacts with Sp1 to form a ternary complex on the Sp1/ERE-A element of the human T-cell leukemia virus type I long terminal repeat (16), and GABPα, which requires a specific interaction with a nonrelated protein, GABPβ, in order to form the $\alpha_2\beta_2$ tetrameric structure which is capable of binding to the GA-rich direct repeats in herpes simplex virus type 1 immediate-early gene promoters (28, 29). The GABPβ protein provides a dimerization motif for the complex and has been shown to interact with GABPα via an ankyrin repeat (65), a structure involved in many protein-protein interactions (34). Interaction with accessory proteins appears likely to be a general mechanism for modulating the DNA binding specificity and/or the transactivating potential of Ets proteins, as is the case in a growing number of other transcription factor families (60).

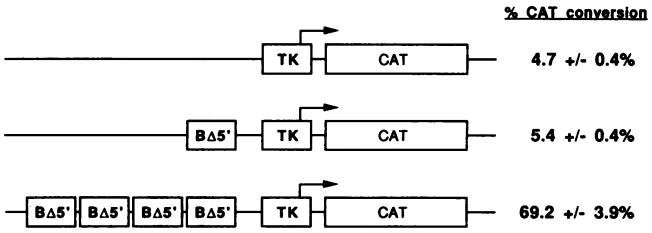

FIG. 8. Analysis of the activating ability of a multimer of λB. A portion of the λB site (BΔ5; see Fig. 1B) was cloned as a monomer and as a tetramer upstream of the TK promoter in TKCAT. These CAT expression constructs were transiently transfected into J558L cells, and CAT activity was measured after 44 h. Results are the average of three independent transfections for each construct and are calculated as the percentage of total [^{14}C]chloramphenicol which is in the acetylated form, reported with a standard deviation.

Multimerization of the λB site produces a strong transactivating element in J558L cells (Fig. 8). This result is similar to that obtained by Pongubala et al. for the related motif in κE3′ (50) but is in contrast to a similar experiment that we have previously described which utilized a distinctly different reporter construct [$(\lambda B)_3OP_\lambda CAT$] that had no activity in J558L cells (55). Differences in the promoters which drive each CAT reporter construct, the specific segment of λB which was multimerized, and the number, organization, and spacing of the λB segments within the multimer may all contribute to the apparently conflicting results which have been obtained. We are currently investigating the possibility that one or more of these differences is responsible for the differential activity of the two reporter constructs.

PU.1 and NF-EM5 appear to be essential for the activity

of both $E_{\lambda 2-4}$ and κE3′. Since these factors are expressed in a cell-type-restricted fashion, it is likely that they are at least in part responsible for the maintenance of the cell-type-specific function of these enhancers. It will be interesting to determine whether they also play an important role in the developmental control of enhancer function. If in fact they do, then one might expect both enhancers to become activated at the same time during B-cell ontogeny, when these factors first exert their effect. Alternatively, other sites, such as λA in $E_{\lambda 2-4}$, could be responsible for controlling enhancer activity in a developmental stage-specific manner. Finally, it will be interesting to determine whether these proteins are in any way involved in the regulation of Ig light-chain gene rearrangement.

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